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VIROLOGICAL STUDY OF LABORATORY INFECTIONS WITH VENEZUELAN EQUINE ENCEPHALOMYELITIS

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In 1938 Beck and Wyckoff (1), and independently from them Kubes and Rios (2), isolated a neurotropic virus from the brains of horses that had died during an epizootic of encephalomyelitis in Venezuela. By immunological and some biological properties it differed from the known viruses of Eastern and Western equine encephalomyelitis, and it was named the Venezuelan equine encephalomyelitis virus.

The Venezuelan equine encephalomyelitis virus is pathogenic for many laboratory animals. Mice are highly sensitive to any method of inoculation. The incubation period in mice that are inoculated intracerebrally equals 36-72 hours. The disease is manifested basically by a general lethargy, paresis, and sometimes paralysis. With an intracerebral inoculation the titer of the virus comprises 10^{10}-10^{12} and, according to the data of separate authors, reaches as high as 10^{18}. Guinea pigs, rabbits and white rats are also highly susceptible to the various methods of inoculation. There are data concerning the susceptibility of monkeys (2, 3). The virus grows well on chick embryos in any method of inoculation, while accumulating in the embryo's different tissues and liquids to a titer of 10^{9} within 18-48 hours, and causes death in a significant portion of the embryos (4, 5). The virus is small; it passes through the Zeiss EK and Berkefeld N filters without noticeably lowering the titer (5, 6).

According to antigenic properties the Venezuelan equine encephalomyelitis virus differs both from the viruses of the Western and Eastern American encephalomyelitis as well as from the other viruses that are known at the present time.

There have been described in the literature several tens of human disease occurrences caused by this virus in natural conditions in South American countries (3, 7, 8). In addition, many communications have been published concerning the disease in laboratory workers (4, 6, 9) and in those who had been inoculated with formol vaccine that was incompletely rendered harmless (10, 11).

Virological and serological investigations of the disease in humans have been conducted rarely, primarily in laboratory infection cases. Investigations have been conducted on blood, nasopharynx washings, cerebrospinal fluid in isolated instances, and on the brain where there had been a fatal termination. Isolation of the virus has been conducted chiefly on mice. Where the virus was received from the blood, from the brain, or from the cerebrospinal fluid, the inoculation is made into the brain; where the isolation was made from a washing, other means of injection have been used, nasal or intraperitoneal.

The virus is isolated regularly from blood and nasopharynx washings on the first to the fifth day of the disease in the first subinoculation. One should note that the virus was detected in the mouth washings from not only the patients that were infected via the aerogenic route, but also from the cases of natural infection, and from those with a subcutaneous inoculation. According to the literary data it is impossible to establish any noticeable difference in the frequency of isolating the virus from the mouth washing or from the blood. According to some authors (10) the times of detecting the virus in the nasopharynx washings coincide with the viremic stages, or are somewhat shorter. In those few cases where the cerebrospinal fluid has been investigated, the virus could not be isolated. In the blood the virus-neutralizing antibodies are manifested rather quickly, but towards the end of the second week they are already detected with certainty.

As a rule the neutralization indices characterizing the humoral immunity after recovery from the disease are very high (from several thousand to several million). Smith et al. consider that the antibody level is in direct proportion to the gravity of the disease. The more serious its course, the higher the humoral indicators.

If in the cases of the serious course of the disease the authors noted no appreciable decrease in the neutralization indices within nine months after the start of the disease, then with the mild forms the antibody level is significantly lowered within as early as five months. Data concerning the ultimate retention times for the antibodies in the blood are nonexistent in the literature. It is noted that after three years from contact with the virus the antibodies in the blood of humans are retained in high titers (12).

Our present work is in relation to a virological study of cases of a disease caused by the Venezuelan encephalomyelitis virus in humans subjected to an accidental aerogenic infection by dried virus as a result of an accident in the laboratory. The epidemiological material is presented in the work of A. N. Slepushkin.

The investigation of the patients was conducted from the 2-5th day of the disease. Blood and mouth washings were taken from all of the patients. In many cases these materials were taken repeatedly for an investigation of the dynamics. Urine and feces were also taken from some of the patients. In all, 87 tests were conducted on the different material from 36 people.

The virus isolation was made through an intracerebral inoculation into white mice weighing 8-10 grams, with three subsequent cerebral subinoculations.

The patients' blood was injected into the brain in a quantity of 0.03 milliliter. The washings from the mouth, the urine and a ten-per cent centrifuged suspension of the feces were treated with penicillin and streptomycin and then were subcutaneously injected into the mice in a 0.03 milliliter quantity. Four to five mice were inoculated in each test. The subinoculations were made after an interval of five days. The observation over the mice of the last subinoculation was conducted for a period of ten days.

In order to prevent the possibility of contact infection, the mice that were infected by the materials from one patient were kept in a separate cage. A special vivarium was established in isolation for the retention of the mice.

In parallel with this, 23 tests were staged on 7-8-day-old chick embryos by inoculating the chorioallantoic membrane with 0.1 milliliter each of the material. For the subsequent subinoculation the brain and the chorioallantoic membrane of the inoculated embryos were homogenized in a jar containing beads in three milliliters of allantoic fluid. The material from each subinoculation was inoculated intracerebrally into mice.

Of the 36 persons examined the diagnosis of Venezuelan equine encephalomyelitis was confirmed laboratorily in 20. Sixteen of these were examined virologically, and in eight cases the virus was isolated. In the remaining cases the etiological diagnosis was established on the basis of serological data.

By an inoculation of chick embryos the virus was isolated from the sample that included the mouth washings from the patients V. A., T. P., and Ye. A.

The results of isolating the virus, according to the time periods of examination, are presented in the illustration. The virus was received from eight patients from the 2-6th day of the disease. Only in one patient (L. V.) was the virus isolated both from the blood (fifth day) and also from the washing (fourth day); in two patients (Z. M. and A. L.) from the blood only; and in five patients (L. I., V. A., T. P., L. I., and Ye A.) from the nasopharynx washings only.

All of the patients, with the exception of one (L. V.), had fever (from 37.5° to 39°C) at the time the materials were taken for investigation.

The negative results received in the attempt to isolate the virus from the blood of the patients V. A., T. P., R. B., L. V., and Ye. A. can be explained by the fact that the blood samples were diluted 3-10 time with a physiological solution when taken.

The great frequency of isolating the virus from the nasopharynx washings could be a logical consequence of the aerogenic method of inoculation and, together with this, of the peculiarities of the disease's pathogenesis.

A comparison of the sensitivity of the methods of isolating the virus showed that the mouse method is more sensitive than that with the chick embryos. In the examination of the same materials by both methods (23 samples) the virus was isolated from six samples on the mice and only one sample on the chick embryos.

Otherwise, in individual cases, urine and fecal tests were investigated at late periods, but always with negative results.

For an identification of the isolated virus there were neutralization experiments staged on the strains isolated from the blood of patient L. V. and from the nasopharynx washing of patient V. A. For these experiments there was used a specific serum that was immune to the Venezuelan equine encephalomyelitis virus. In both cases the neutralization indices were approximately 100,000, which corresponded to the serum titer in the proportion that the standard strain of the Venezuelan equine encephalomyelitis virus corresponds to that indicated on the label.

The pathogenicity of the isolated strains were also tested on some of the laboratory animals and chick embryos for the purpose of identifying the virus.

The virus is pathogenic for mice in the various methods of inoculation. The titer of the virus in an intracerebral inoculation of mice amounted to from 10^8 to 10^{11} in the various experiments.

The possibility of contact inoculation was proved in special experiments where fresh mice were placed into cages with inoculated animals. This emphasizes the necessity of observing a strict regime in the conduct of investigations with Venezuelan equine encephalomyelitis.

Strain No. 2 was intracerebrally inoculated into two rabbits weighing two kilograms, two guinea pigs weighing 300 grams, and four white rats weighing 80-100 grams. Within thirty hours there was noted in the guinea pigs lethargy, slight mobility, and an increase in temperature to 42°C. Death came in 48-72 hours. In the rabbits a fever appeared within 24 hours, and on the fifth 24-hour period they died. The disease was developed latest of all in the white rats - on the fifth to the seventh day. Tetraplegia was observed. All of the rats died on the 6-9th day. The virus multiplied actively on the chick embryos. In an inoculation of 7-8-day-old embryos on the chorioallantoic membrane, with the virus in a dilution of 10^3 and 10^4, multiplication of the virus was registered in a high titer within 18 hours.

Thus according to the antigenic properties and the pathogenicity for animals the strains isolated from the patients proved to be identical to the Venezuelan equine encephalomyelitis virus.

We also conducted serological investigations for the purposes of studying the dynamics of the accumulation of antibodies in the blood of the convalescents, the development of cases of clinically unrecognized sicknesses, and the possibility of sickness in the people having had contact with the patients or having worked with the virus.

Blood was taken from the patients, where possible, three times: during the first week of the disease, towards the end of the second week, and after 25-40 days.

The neutralization experiments were staged on white mice weighing 8-10 grams. Mixtures of the sera with equal amounts of increasing tenfold dilutions of the Venezuelan equine encephalomyelitis virus (the strain that was isolated from Patient V.) were placed into a thermostat. After one hour the contents of each test tube, a volume of 0.03 milliliter, was injected intracerebrally into four mice. An account of the test's results was conducted for a period of ten days, after which the surviving animals were destroyed. The resultant data was subjected to the usual statistical processing for determination of the neutralization indices.

In the table are shown the dynamical results of the serological investigations of sera from the twenty patients in whom the diagnosis of Venezuelan equine encephalomyelitis had been confirmed. Out of the thirteen sera taken during the first week of the disease, in nine no antibodies were detected; in three cases diagnostically verified neutralization indices were received. The indicators of the neutralizing capacity of one of the sera were doubtful. Toward the end of the second week the serological diagnosis could already be established in fifteen of the seventeen cases that were examined at these times, whereupon the neutralization indices of half of the examined sera exceeded 1,000. By the 25-40th day of the disease the antibodies in the blood of the convalescents attained a very high level. The neutralization indices of fourteen of the eighteen sera investigated comprised 10,000 and higher. A serological diagnosis of the sickness as Venezuelan equine encephalomyelitis could be established by this time in all twenty cases. Taking the early appearance of the antibodies in the blood into consideration, in conducting a serological diagnosis of Venezuelan equine encephalomyelitis by means of neutralization experiments, as a rule, it is possible to confine oneself to an investigation of the pair of sera, which are taken at the start of the disease and at the end of the second week. With negative and questionable results, however, it is necessary to investigate a third serum sample taken after 4-5 weeks.

It was interesting to compare the dynamics of the increase of antibodies with the disease's clinical form. In order to facilitate the conduct of the indicated parallels, all data in the table are placed, to the degree possible, in the order of the antibodies' level of elevation, chiefly in the first two weeks of the disease (As has been shown, by the 4-5th week there occurred a certain equalization of the antibody levels.) A comparison of the dynamics of the increase in the antibodies with the disease's clinical form shows that there was no direct and constant relation between them. Forms of various gravity are found both in the upper and in the lower halves of the table's column.

As mentioned above, there was also investigated by the serological method the large group of people having had contact with the patients, those having worked with the virus, and also those persons who complained of indisposition in the two-week period after the accident in the laboratory. A total of 39 such persons were examined, with negative results.

Conclusions

1. Virological and serological examinations were conducted on 63 people, the patients with Venezuelan equine encephalomyelitis, the persons who had been in contact with the patients, and those having worked with the indicated virus. Thirty-six persons were examined by serological and virological methods, and 27 were examined by serological methods alone.

2. Isolation of the virus was conducted on white mice and chick embryos. From the 36 persons 87 different samples of blood, mouth washings, urine and feces were examined for the virus. The virus was detected in eight patients on the 2-6th day of the disease. Three strains were isolated from the blood, and six strains from the mouth washings.

3. In the neutralization test 99 blood sera, taken from 63 persons, were examined. The blood sera from the convalescents were investigated dynamically 2-3 times. Antibodies to the Venezuelan equine encephalomyelitis virus were observed in twenty persons, with which all of them endured the disease. There were no cases observed of an asymptomatic course of the infection in those persons having had contact with the patients, or in those having worked with the virus. Antibodies were detected in the convalescents in a sufficiently high titer by the 10-12th day of the illness.

4. As a result of complex virological and serological investigations a diagnosis of Venezuelan encephalitis was confirmed in twenty persons.

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THE RESULTS OF VIROLOGICAL EXAMINATION OF THE PATIENTS WITH VENEZUELAN EQUINE ENCEPHALOMYELITIS

PATIENTS' INITIALS	MATERIAL INVESTIGATED	DAY MATERIAL WAS TAKEN														OTHER MATERIAL INVESTIGATED
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
L.L.	Blood			●	●											feces, 13th day
	Washing		■													
V.V.	Blood				●										●	
	Washing				■										●	
T.K.	Blood		●									●				
	Washing		■										●			
P.K.	Blood				●										●	feces, 14th day
	Washing				●										●	
Z.L.	Blood					■									●	urine, 15th day
	Washing														●	
L.K.	Blood									●						urine 7th day
	Washing														●	
A.L.	Blood					■										
	Washing													●		
L.N.	Blood					■									●	feces, 14th day
	Washing				■										●	
Z.R.	Blood						●							●		
	Washing						●								●	
L.P.	Blood						●								●	
	Washing						■								●	
G.I.	Blood					●									●	
	Washing														●	
Ye.G.	Blood					●										
	Washing														●	
I.K.	Blood									●						
	Washing														●	
Ye.K.	Blood				●											
	Washing				■											
P.M.	Blood					●									●	
	Washing														●	
R.S.	Blood							●							●	
	Washing							●							●	

● Virus Not Isolated ■ Virus Isolated

TABLE

Dynamics of the growth of antibodies to the Venezuelan equine encephalomyelitis virus in the blood of the recovered patients.

Initials of Patient	The Day Blood was Taken			The Clinical Form of the Disease
	3-7th	12-14th	25th-40th	
	Neutralization Indices			
R.S.	0	14	10,000	Severe
I.K.		14	>10,000	Severe
M.P.		67	>10,000	Mild
M.P.	0	100	100	Mild
Ye.K.	0	100	1,000	Mild
P.M.	0		1,000	Median Gravity
R.S.			3,162	Mild
Z.R.	0	213	31,620	Median Gravity
R.K.	0	316		Median Gravity
L.P.		467	21,380	Median Gravity
G.K.	0	1,000	10,000	Severe
Ye.G.	0	1,000	10,000	Severe
T.K.		1,000	100,000	Median Gravity
L.N.	0	1,000	100,000	Median Gravity
S.T.	46		10,000	Median Gravity
A.L.	79	10,000	10,000	Median Gravity
Z.L.	218	10,000	10,000	Mild
V.V.		4,677	>10,000	Mild
L.K.		>10,000		Severe
L.D.	148	>10,000	>10,000	Median Gravity